

## ISOLATION AND CHARACTERISATION OF TWO THERMOTOLERANT *KLUYVEROMYCES* YEAST STRAINS

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### Summary

*Two new thermotolerant lactose consuming yeast strains, producers of thermostable superoxide dismutase (SOD) (EC: 1.15.1.1.) were isolated and taxonomically identified as Kluyveromyces marxianus var. lactis T1 and Kluyveromyces marxianus var. bulgaricus T3. The influence of carbon source and growth temperature on the specific growth rate and SOD biosynthesis was also studied. Both strains grew well on lactose and/or lactose containing feedstocks and those substrates could be used for the production of SOD enzyme with high activity. Temperature of 40°C was proved to be suitable for cultivation of both strains with respect of SOD biosynthesis. Assay for thermostability of SOD enzyme of T1 and T3 strains cultivated on lactose shows that it is a thermostable one.*

*The newly isolated and identified strains can be used as producers of thermostable SOD when grown on lactose and lactose containing feedstocks at elevated temperatures.*

### Introduction

At present there is a considerable interest in employing yeasts for production of commercially important enzymes. It is well known that the cell yield and growth temperature are major factors influencing the cost of enzymes of microbial origin [12]. Thus, a rational strategy is the selection of thermophilic and thermotolerant strains-producers of thermostable enzymes when grown on low-costing substrates.

Yeasts are a good alternative for the production of the enzyme superoxide dismutase (SOD). This enzyme is of vital importance for oxygen tolerance of the living organisms. Recently its practical application is greatly enlarged due to its catalytic function – dismutation of toxic oxygen intermediates in cases when their scavenging is essential [3, 5, 8].

The work presented describes the isolation of two thermotolerant lactose consuming yeast strains, producers of SOD, and their taxonomical identification. The

influence of the carbon source and the growth temperature on specific growth rate, the SOD biosynthesis and activity are also studied.

## Materials and Methods

**Isolation of microorganisms.** Eighty-five samples from different dairy products were used for the isolation of thermotolerant lactose consuming yeasts. The isolation nutrient medium contained (g/l):  $(\text{NH}_4)_2\text{SO}_4$  - 3.0;  $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$  - 0.7; NaCl - 0.5;  $\text{CaCl}_2$  - 0.4;  $\text{KH}_2\text{PO}_4$  - 1.0;  $\text{K}_2\text{HPO}_4$  - 0.1 and lactose - 20.0; supplemented with 1% yeast extract and pH adjusted to 4.5. Penicillin – 6.2 mg/l and streptomycin – 20 mg/l were also added for limitation of the bacterial growth.

All the samples were submitted to the following procedure for isolation and characterisation: the sample, mechanically homogenised with 5 – 10 ml nutrient medium, was added to 100 ml nutrient medium in 500 ml Erlenmeyer flasks and cultivated for 48 h at 40°C on rotary shaker at 220 rpm. Those of the samples showing good growth were streaked on agar plates containing malt agar, and pure yeast cultures were isolated from the obtained single colonies. These cultures were once again tested for growth at 45°C on lactose as a single carbon and energy source. Two of the isolates were chosen for further characterisation after this procedure.

**Taxonomic determination.** These two isolated yeast strains were identified taxonomically according to the methods and criteria of Kreger Van Rij [6] and Barnett et al. [1] using Difco nutrient media (Difco Laboratories, Detroit, USA) and chemicals of analytical quality. Yeast type cultures were used as referent ones.

**Cultivation of microorganisms.** Batch cultures at different growth temperatures (30 and 40°C) were obtained on Rider medium [9]

with different carbon sources and on a nutrient medium prepared on the basis of whey as follows: the whey was submitted to ultrafiltration (DDS membranes 5 KD) and the obtained filtrate was diluted with distilled water to lactose concentration up to 2.5%.  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{K}_2\text{HPO}_4$  were also added to final concentrations of 3.0 and 0.1 g/l respectively.

**Analytical methods.** Cell dry weight was determined gravimetrically after drying washed cells to constant weight at 105°C. Glucose growth yield values ( $Y_s$ ) were calculated as g cells dry weight per g glucose consumed.

Crude extract preparation. The cell biomass was harvested by centrifugation at 4500 rpm for 10 min, triply washed (twice with distilled water and once with 0.05 M potassium phosphate buffer pH 7.8) and mechanically disrupted on disintegrator FUG2.

Protein concentration was determined according to the method of Lowry et al. [7]. Bovine serum albumin was used as a standard.

SOD assay was performed according to the method of Beauchamp and Fridovich [2]. One unit of activity was expressed as the amount of cell-free protein, which caused 50% inhibition to the reduction rate of nitro blue tetrazolium to blue formazan.

Enzyme thermostability assay. The thermostability was expressed as a percentage residual SOD activity at a given temperature for a defined interval of time, compared to the untreated control which activity was accepted as 100 % [12].

## Results and Discussion

Two yeast strains were isolated and taxonomically identified as *Kluyveromyces marxianus* var. *lactis* T1 and *Kluyveromyces marxianus* var. *bulgaricus* T3. Their cell morphology and cultural characteristics are presented in Table 1. Strain T1 possessed round cells which reproduced by monopolar

budding on narrow base. Strain T3 cells were also round and reproduced by monolateral budding. Both strains formed ascospores (1 - 4 spherical spores in the form of tetrad or a chain), but neither formed pseudomycelium and chlamidospores, nor produced pigments.

Table 1. Morphological and cultural characteristics of T1 and T3 strains.

Characteristics	<i>Kl. marxianus</i> var. <i>lactis</i> T1	<i>Kl. marxianus</i> var. <i>bulgaricus</i> T3
Source	skim curd	yellow cheese
Cell morphology		
Shape and form		
Broth <sup>a</sup>	round, singly or in pairs	round
Agar <sup>a</sup>	as broth	as broth
Size (µm)		
Broth <sup>a</sup>	(0.1-0.2)x(0.2-0.3)	(0.05-0.1)x(0.1-0.2)
Agar <sup>a</sup>	as broth	as broth
Cultural characteristics		
Broth culture	sediment formation	sediment formation
Streak culture	smooth, soft, white coloured colony; slightly shiny with smooth edge	smooth, soft, white coloured colony; slightly shiny with lifted center, smooth edge and concave form

<sup>a</sup> - malt yeast broth and agar

The strains' physiological properties are presented in Table 2. It was evident that both strains were auxotrophs (deficient in nicotinic acid), did not assimilate nitrates, but split arbutin. The test for resistance to high concentrations of NaCl indicated that strain T1 grew well on 3% NaCl, while strain T3 showed sufficient growth even on 7% NaCl. Concerning the growth temperature requirements, the results presented clearly showed that both strains were thermotolerant ones (Table 2).

The ability of the strains to grow on different substrates and the type of their utilisation revealed also some differences

(Table 3). The assimilation and fermentation tests performed showed that strain T1 assimilated maltose, cellobiose and glycerol while strain T3 could not consume these substrates aerobically. It assimilated D, L lactic acid while strain T1 did not. The major difference in the catabolic activities of the strains was the ability of strain T1 to utilise lactose both by fermentation and respiration while strain T3 could not catabolise this disaccharide by fermentation. Thus, a phenomenon of Kluyver effect occurred in strain T3 because it was able to ferment the monosaccharides constructing lactose (glucose and galactose) [10].

Table 2. Physiological properties of T1 and T3 strains.

Property	T1	T3
Nitrate utilisation	No	No
Growth without vitamins	No, requirement of nicotinic acid	No, requirement of nicotinic acid
Acid production	Yes	Yes
Growth at different T (°C)		
minimal	5–6	5–6
optimal	40	40
maximal	50	48
Growth on 50% D-glucose		
on the 3 <sup>rd</sup> day	No	No
on the 17 <sup>th</sup> day	No	No
Splitting of arbutin		
on the 3 <sup>rd</sup> day	Yes	Yes
on the 17 <sup>th</sup> day	Yes	Yes
Growth in media of high osmolarity	Yes, 3% NaCl	Yes, 7% NaCl

In such a way, two new thermotolerant yeast strains, consuming lactose were isolated and identified as species *Kl. marxianus* var. *lactis* and species *Kl. marxianus* var. *bulgaricus* on the basis of morphological characteristics, physiological properties and biochemical activities.

*Kluyveromyces* yeasts are among those of industrial significance due to their ability to grow on lactose and lactose containing feedstocks, producing vitamins and enzymes [4, 11].

The ability of the two newly isolated thermotolerant strains to grow on lactose and the influence of this carbon source on the SOD enzyme activity was studied. Batch cultures of T1 and T3 strains on Rider medium with 2 % lactose at 30°C represented

values for maximal specific growth rate ( $\mu^{\max}$ ) of 0.435 h<sup>-1</sup> and 0.456 h<sup>-1</sup> respectively. These results showed that both strains grew well on lactose. Data of particular interest are those concerning the biosynthesis of the enzyme SOD. In Table 4 the values for the specific enzyme activity of T1 and T3 strains cultivated on different carbon sources are depicted. It was evident that the highest values for SOD activity were found on lactose and whey. Thus, these substrates could be used as suitable carbon sources for production of SOD with high activity.

As it is mentioned above both strains are thermotolerant ones. Due to this fact the influence of the growth temperature on the cell growth and SOD activity was examined.

Table 3. Fermentation and assimilation of different carbon sources by T1 and T3 strains.

	T1	T3		T1	T3
C-Source	Fermentation		C-Source	Fermentation	
D-Glucose	+	+	Maltose	-	-
D-Galactose	+	+	Lactose	+	-
Sucrose	+	+	Melibiose	+	+
C-Source	Assimilation		C-Source	Assimilation	
D-Glucose	+	+	Ribitol	-	-
D-Galactose	+	+	Galactitol	-	-
Sucrose	+	+	D-Manose	-	-
Maltose	+	-	D-Glucitol	-	-
Cellobiose	±	-	$\alpha$ -CH <sub>3</sub> - $\beta$ -D-Glucoside	-	-
$\alpha,\alpha$ -Trehalose	-	-	Salicine	-	-
Lactose	+	+	D, L-Lactate	-	+
Melibiose	-	-	Starch	-	-
Raffinose	+	+	D-Xylose	+	+
L-Sorbose	-	-	L-Arabinose	+	+
Melezitose	-	-	Succinate	±	±
Inulin	+	+	D-Arabinose	-	-
L-Rhamnose	-	-	D-Ribose	-	-
Ethanol	+	+	Citrate	-	-
Glycerol	+	-	<i>myo</i> -Inositol	-	-
Erythritol	-	-	Methanol	-	-
Control	-	-			

Table 4. Dependence of T1 and T3 SOD activity on different carbon sources.

C-Source	SOD activity (U/mg of protein)	
	T1	T3
Glucose	6–10	5–11
Galactose	40–50	45–60
Lactose	70–75	95–100
Xylose	55–60	55–66
Ethanol	50–55	45–50
Whey	68–75	79–85

Batch cultures on glucose were obtained as described in Materials and Methods at growth temperatures of 30° and 40°C. The yield coefficients obtained were 0.44 – 0.45 + 0.01 and 0.42 – 0.43 + 0.02 respectively. The averaged activity of the SOD enzyme measured at the end of exponential growth phase was 7 - 10 + 0.3 U/mg of protein. These results indicated that the activity of the SOD enzyme was not influenced by higher growth temperature.

The effect of temperature on SOD activity was also evaluated by measuring the enzyme thermostability using crude enzyme homogenates. For this purpose, batch cultures grown on lactose were prepared and the activity of the SOD enzyme at 70°C for

30 min was assayed as described in Materials and Methods. The values obtained for residual enzyme activity were 63.3% and 69.5% for T1 and T3 respectively indicating a thermostable enzyme.

All these data show that the newly isolated and taxonomically identified strains *Kluyveromyces marxianus* var. *lactis* T1 and *Kluyveromyces marxianus* var. *bulgaricus* T3 (National Bank for Industrial Microorganisms and Cell Cultures № 1984) can be used as producers of a thermostable superoxide dismutase when grown on lactose (whey) at elevated temperatures. Being thermotolerant (growth optimum 40°C) they also can simplify heat loss problems in large-scale bioreactors.

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